AN ESSENTIAL METHIONYL RESIDUE IN THE *lac*-PERMEASE OF *E. coli*

Joseph YARIV and Pnina ZIPORI

Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

Received 30 May 1972

1. Introduction

Cowie et al. [1] have shown that in the protein of a methionine auxotroph of *E. coli* grown in the presence of norleucine partial replacement of methionine by norleucine occurs. The finding that replacement of methionine was uniform in different protein fractions, obtained by chromatography on DEAE-cellulose of the *E. coli* protein, was interpreted to mean that the probability of substitution of methionine by norleucine at each methionine locus in a polypeptide chain is the same. β-Galactosidase modified by partial incorporation of norleucine was found to be enzymatically active. Similarly, β-galactosidase activity was preserved when all methionine of the enzyme was replaced with selenomethionine [2].

Recently, it has been shown that the site-directed reagent, *N*-bromoacetyl β-D-galactopyranosylamine, inactivates β-galactosidase by alkylating a methionyl residue near the active site of the enzyme [3]. When β-galactosidase is induced in a methionine auxotroph of *E. coli* grown on methionine, the enzyme can be fully inactivated by the above reagent. However, enzyme induced in this strain in the presence of norleucine is only partially inactivated by this site-specific reagent, since the norleucyl residue which replaces the methionyl residue at the active site of the enzyme cannot be alkylated [4]. The residual activity of norleucine-modified enzyme treated with *N*-bromoacetyl galactosylamine thus measures methionine replacement at a specified methionine locus of β-galactosidase and therefore, according to the argument presented above, at any methionine locus of newly synthesized protein.

The objective of the study to be reported here was to determine the quantitative relationship between methionine replacement by amino acid analogs and permease function. We find that partial introduction o norleucine into a single methionine locus in the *lac*-permease protein causes a corresponding decrease in the transport of *o*-nitrophenyl β-D-galactopyranoside (ONPG). *lac*-Permease carrier function is preserved when all methionine is replaced with selenomethionine but the modified permease is inhibited in the course of ONPG hydrolysis. We conclude that a methionyl residue is part of the substrate binding site of *lac*-permease.

2. Methods

2.1. *Bacterial cultures*

A methionine auxotroph of *E. coli* K12 was used. The strain, obtained by the courtesy of Dr. Y.S. Halpern of the Hebrew University, Jerusalem (strain CS-8 of his collection), is a *lac*-operon inducible ($i^+ z^+ y^+$) and a met A^- strain. Bacteria were grown in a synthetic medium containing per litre: 13.6 g KH_2PO_4; 2.0 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4 \cdot 2H_2O$ and 0.5 mg $FeSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7 with KOH. Glycerol, 4 g/l, served as a carbon source. The cultures grown with aeration at 37° and with added L-methionine, 25 mg/l, were harvested in the exponential phase at a density of approx. 100 Klett units (filter 66). Bacteria were sedimented in a refrigerated centrifuge, washed once and resuspended in the salts solution.

Norleucine cultures were grown as above by inoculating the resuspended bacteria into the synthetic medium supplemented with L-methionine, 2.5 mg/l, and with DL-norleucine, 500 mg/l. In parallel, growth on full medium containing 25 mg L-methionine/l was initiated with an inoculum of the same size as in the

North-Holland Publishing Company – Amsterdam

norleucine culture. After each culture had grown to a preselected density, isopropyl thio-β-D-galactoside (IPTG), 5×10^{-4} M, was added as inducer. Growth was continued till about twice the density at induction and bacteria were sedimented, washed, and resuspended in a 0.1 M sodium phosphate buffer, pH 7.0, containing chloramphenicol, 50 μg/ml.

Selenomethionine cultures were grown as were the norleucine cultures and were also accompanied by a parallel growth on full medium. In this case DL-selenomethionine was added to a concentration of 66 mg/l and no methionine was added. In contrast to the "logarithmic" growth on selenomethionine of the ML strain used by Cowie and Cohen [2], the strain used here gave a "linear" growth on selenomethionine. The same schedule of induction by IPTG and harvesting was followed as in the norleucine cultures. The washed cells were used in the *lac*-permease and β-galactosidase assays.

Density of the bacterial suspensions was calculated from absorbance at 650 nm. $A^{1\,cm}_{650\,nm}$ of 1.0 was taken as corresponding to 10^9 cells/ml.

2.2. *Assay of permease*

lac-Permease-mediated flux of ONPG was used to measure permease activity. Flux was measured at a saturating concentration of ONPG, which was found to be 2×10^{-2} M, and was corrected for nonspecific flux of ONPG, which is the flux persisting in 2×10^{-2} M ONPG in the presence of 2×10^{-1} M melibiose, this being a saturating concentration of the competitive inhibitor. Flux was measured as moles of *o*-nitrophenol (ONP) released in *E. coli* cells by β-galactosidase.

The rate of ONP released by a known quantity of bacteria was determined in a Cary 14 recording spectrophotometer at 420 nm in a cell of 1 cm light path kept at 30 ± 0.5°. The assay was conducted in a 0.1 M sodium phosphate buffer at pH 7.0. To determine the *o*-nitrophenol concentration, the molar absorbance, $\epsilon_{420\,nm}$, of 5200 was used for the *o*-nitrophenolate ion and the concentration of *o*-nitrophenol at pH 7.0 was calculated by assuming a pK_a of 7.2.

2.3. *Assay of β-galactosidase*

β-Galactosidase content of *E. coli* cells was determined by the procedure of Pardee et al. [5]. This consists of lysis of a known quantity of bacteria by incubation with toluene for 30 min at 37° and measurement of the rate of hydrolysis of ONPG by the released enzyme at 28°. Reaction is stopped by addition of sodium carbonate. Absorbance of *o*-nitrophenolate at 420 nm was determined spectrophotometrically. In order to conform with the assay procedure, the quoted molar absorbance of 7500 was used to determine *o*-nitrophenolate concentration.

2.4. *Inactivation of β-galactosidase*

β-Galactosidase was released by sonic disruption (Branson sonifier) of a suspension of *E. coli* cells, approx. 3×10^{10} cells/ml, in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.001 M $MgCl_2$. The clear supernatant obtained by centrifugation of the disrupted cells for 30 min at 15 000 rpm in a refrigerated Servall centrifuge was used. Inactivation of β-galactosidase by incubation of the extract with *N*-bromoacetyl β-D-galactopyranosylamine was carried out essentially as described [3]. Concentration of the site-specific reagent in the reaction mixture was 7.4×10^{-3} temperature was 35°. In following the time course of the inactivation 15 to 25 μl samples were transferred from the reaction mixture to 1 ml of the assay solutio of the following composition: 0.7 g ONPG/l; 0.1 M sodium phosphate, pH 7.0; 0.001 M $MgCl_2$ and 0.1 M 2-mercaptoethanol. Rate of release of *o*-nitrophenol at 28° was determined in a Cary recording spectrophotometer at 420 nm.

3. Results

β-Galactosidase in extracts obtained from *E. coli* grown on methionine is fully inactivated by the galactosyl reagent (fig. 1). In experiment 1 induction of *lac*-operon with IPTG was started at cell density corresponding to 25–30 Klett units and the cells were harvested after approximately one generation at 50–70 Klett units. In experiment 2 cells were induced at a density of 40–47 Klett units and harvested at 80–107 Klett units. The extracts obtained from *E. coli* grown in the presence of norleucine are only partially inactivated by the galactosyl reagent, the residual activity being 19% in experiment 1 and 51% in experiment 2 (fig. 1). *lac*-Permease and β-galactosidase activities of the two norleucine cultures and their control cultures on methionine are given in table 1. *lac*-Permease activities and methionii

Table 1
The effect of methionine replacement by norleucine on β-galactosidase and *lac*-permease activities of *E. coli*.

E. coli grown on:	*lac*-Permease activity of 1×10^9 cells, nmoles ONP/1 min	β-Galactosidase content of 1×10^9 cells, nmoles ONP/1 min	Fraction of β-galactosidase activity resistant to alkylation
Experiment 1			
Methionine	207	840	0.00
Norleucine	134	670	0.19
Experiment 2			
Methionine	238	780	0.00
Norleucine	66	430	0.51

replacement of the two norleucine cultures are compared in table 2. *lac*-Permease activity is normalized to the β-galactosidase content of the respective cultures and expressed activity per cent of the *lac*-permease of the control culture grown on methionine. For example, in experiment 1, *lac*-permease activity of the norleucine cultures (see table 1), 134 nmoles ONP/min/1 $\times 10^9$ cells, is multiplied by the ratio of the β-galactosidase activities of the two cultures, $\frac{840}{670}$, to give a normalized activity of 168 nmoles ONP/min/1 $\times 10^9$ cells. This normalized value of permease activity of the norleucine culture corresponds to 81% of permease activity of the control methionine culture whose activity is 207 nmoles ONP/min/1 $\times 10^9$ cells. The measure of methionine replacement by norleucine is the fraction of β-galactosidase activity which is resistant to treatment with *N*-bromoacetyl galactosy amine.

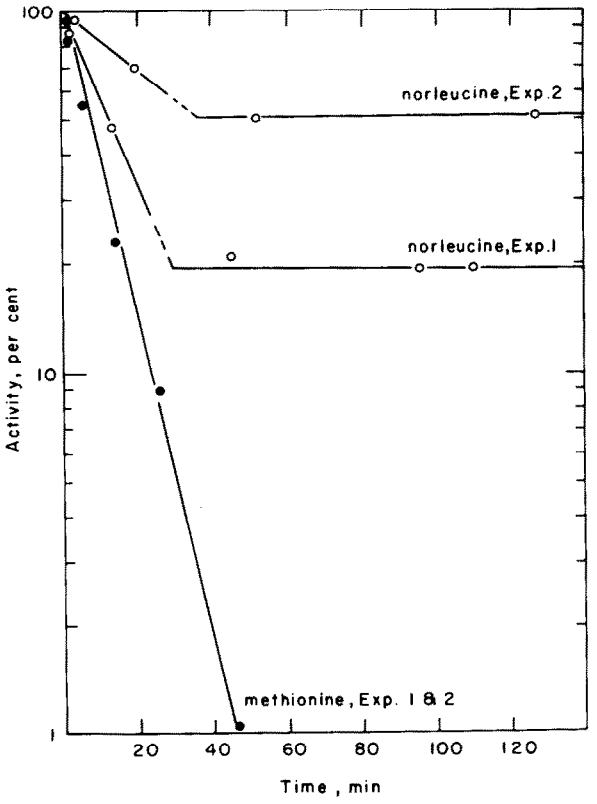

Fig. 1. Inactivation of β-galactosidase by *N*-bromoacetyl galactosylamine. The full circles are for extracts of *E. coli* grown on methionine, the open circles for extracts of *E. coli* grown on norleucine.

When *lac*-permease activity of cells grown on selenomethionine was determined as described in Methods, namely in a recording spectrophotometer with 2×10^{-2} M ONPG as substrate, a decrease in the rate of ONPG hydrolysis during the time of the assay was observed (fig. 2). Normal cells, that is cells grown on methionine, give zero order kinetics of ONPG hydrolysis under such conditions. Zero order kinetics was also observed with cells modified by selenomethionine when a different chromogenic substrate, *p*-nitrophenyl β-D-galactopyranoside (PNPG) was

Table 2
Comparison of normalized *lac*-permease activity of *E. coli* grown on norleucine with methionine replacement.

Experiment	*lac*-Permease activity (%)	Methionine replaced (%)
1	81	19
2	50	51

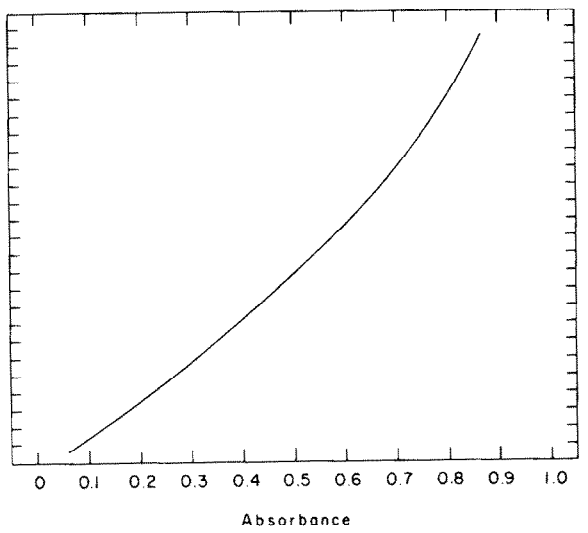

Fig. 2. Time course of ONP release by selenomethionine-modified *E. coli* cells. The curve represents the Cary spectrophotometer tracing of absorbance at 420 nm on a chart moving in the vertical direction at a rate of 5 divisions/min. The sample cell contained 6.8×10^8 cells in a volume of 1 ml. Concentration of ONPG was 2×10^{-2} M in 0.1 M sodium phosphate, pH 7.0.

used at a comparable concentration, 4×10^{-2} M, or when assay was done at one tenth the usual ONPG concentration, namely at 2×10^{-3} M. Subsequent experiments have established that this inhibition of *lac*-permease carrier function in selenomethionine-modified cells is a complex one. It occurs whenever *o*-nitrophenol and a relatively high concentration of a nitrophenyl-containing substrate, such as ONPG or PNPG, are present in the reaction mixture. β-Galactosidase obtained from cells grown on selenomethionine shows zero order kinetics of ONPG hydrolysis even at high ONPG concentration. The *lac*-permease activity of selenomethionine-modified cells, as determined by hydrolysis of PNPG, corresponds to about 90% activity of normal cells.

The following observations about the selenomethionine-modified *lac*-operon proteins of *E. coli* are also of interest: Selenomethionine-modified β-galactosidase is completely inactivated by *N*-bromoacetyl galactosylamine with a rate of inactivation which is at least ten times faster than the rate of inactivation of normal β-galactosidase. The *lac*-permease of selenomethionine-modified cells is inhibited by *N*-ethylmaleimide with the same rate as that of norma cells.

4. Discussion

The data in table 1 suggest more than one possible correlation between the extent of methionine replacement and the level of the β-galactosidase and of the *lac*-permease activities.

One possibility is to compare the levels of either β-galactosidase or *lac*-permease in *E. coli* cells grown on norleucine to the levels of these two functions in cells grown on methionine. The reduced level of β-galactosidase is correlated with replacement of a single methionyl residue according to the following formula

$$\frac{\beta\text{-galactosidase}^{nor}}{\beta\text{-galactosidase}^{met}} = 1 - f$$

where f is the fraction of methionine replaced by norleucine as measured by the fraction of β-galactosidase activity resistant to alkylation with *N*-bromoacetyl galactosylamine. By such a procedure the reduced level of permease fits the following formula

$$\frac{\text{permease}^{nor}}{\text{permease}^{met}} = (1-f)^2$$

which corresponds to destruction of permease by replacement of either of two essential methionines. However, there is no direct evidence that there is a methionine essential for the functioning of β-galactosidase whose replacement by norleucine causes complete loss of catalytic activity. The observe correlation between methionine replacement and the reduced level of β-galactosidase could reflect a general reduction of protein synthesis in cells grown in the presence of norleucine.

Normalization of *lac*-permease level to that of β-galactosidase seems well justified in view of their being products of neighbouring genes of a common operon. In table 2 *lac*-permease activity of the two *E. coli* cultures grown in the presence of norleucine (see table 1) is normalized to the respective levels of β-galactosidase and expressed as activity per cent of the corresponding *E. coli* grown on methionine. It car

be seen that loss of permease activity is strictly correlated with methionine replacement. This one-to-one correspondence indicates that replacement of a single methionyl residue in the *lac*-permease protein abolishes transport. The fact that selenomethionine can replace methionine without loss of permease activity whereas norleucine cannot suggests that the essential methionine is not a structurally but rather a chemically important residue. The mechanism of the observed inactivation of the selenomethionine-modified *lac*-permease by ONPG is not understood. However, its occurrence strongly suggests that the effect must occur on a strategically located selenomethionine. A likely choice for its location would be the substrate-binding site of the permease molecule.

Acknowledgement

We thank A. Joseph Kalb for his comments.

References

[1] D.B. Cowie, G.N. Cohen, E.T. Bolton and H. de Robichon-Szulmajster, Biochim. Biophys. Acta 34 (1959)
[2] D.B. Cowie and G.N. Cohen, Biochim. Biophys. Acta 26 (1957) 252.
[3] J. Yariv, K.J. Wilson, J. Hildesheim and S. Blumberg, FEBS Letters 15 (1971) 24.
[4] F. Naider, Z. Bohak and J. Yariv, Biochemistry, in press.
[5] A.B. Pardee, F. Jacob and J. Monod, J. Mol. Biol. 1 (1959) 165.